

Alterations in Neural Crest Migration by a Monoclonal Antibody that Affects Cell Adhesion

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ABSTRACT The possible role of a 140-kD cell surface complex in neural crest adhesion and migration was examined using a monoclonal antibody JG22, first described by Greve and Gottlieb (1982, *J. Cell. Biochem.* 18:221–229). The addition of JG22 to neural crest cells in vitro caused a rapid change in morphology of cells plated on either fibronectin or laminin substrates. The cells became round and phase bright, often detaching from the dish or forming aggregates of rounded cells. Other tissues such as somites, notochords, and neural tubes were unaffected by the antibody in vitro even though the JG22 antigen is detectable in embryonic tissue sections on the surface of the myotome, neural tube, and notochord. The effects of the JG22 on neural crest migration in vivo were examined by a new perturbation approach in which both the antibody and the hybridoma cells were microinjected onto neural crest pathways. Hybridoma cells were labeled with a fluorescent cell marker that is nondeleterious and that is preserved after fixation and tissue sectioning. The JG22 antibody and hybridoma cells caused a marked reduction in cranial neural crest migration, a build-up of neural crest cells within the lumen of the neural tube, and some migration along aberrant pathways. Neural crest migration in the trunk was affected to a much lesser extent. In both cranial and trunk regions, a cell free zone of one or more cell diameters was generally observed between neural crest cells and the JG22 hybridoma cells. Two other monoclonal antibodies, 1-B and 1-N, were used as controls. Both 1-B and 1-N bind to bands of the 140-kD complex precipitated by JG22. Neither control antibody affected neural crest adhesion in vitro or neural crest migration in situ. This suggests that the observed alterations in neural crest migration are due to a functional block of the 140-kD complex.

Several important developmental events are accompanied by temporal or spatial changes in the composition of the extracellular matrix (ECM).¹ For example, hyaluronate accumulates in the corneal stroma during the invasion of the corneal fibroblast and is subsequently removed by increased production of hyaluronidase (25). Fibronectin has been implicated in guiding cell movement during gastrulation (1) and neural crest migration (10, 24) because it lines pathways followed by migrating cells. Such correlations have led to the proposal that adhesive interactions between the extracellular matrix and the embryonic cell surface may play an important role in morphogenesis (13).

The neural crest is an interesting model for examining the role of cell surface–ECM interactions because these cells migrate extensively during development along pathways that

are lined with ECM molecules. Neural crest cells arise during neurulation and depart from the newly formed neural tube shortly after tube closure. The pathways followed by neural crest cells contain numerous matrix molecules including fibronectin, hyaluronic acid, type I collagen, and probably various other molecules. In tissue culture, neural crest cells can migrate in a directed fashion along fibronectin rich matrices (22) and can also migrate on laminin (17). When transplanted into premigratory regions of axolotl embryos, microcarrier filters coated with embryonic matrices from older embryonic regions can promote premature migration of neural crest cells (14). These studies indicate that the ECM may be both permissive and stimulatory for neural crest migration.

Recently, several monoclonal antibodies were described that recognize cell surface proteins involved in adhesion (11, 16, 20). Two of these monoclonals, called JG22 and CSAT, perturb the adhesion of embryonic muscle cells to some

¹ Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimyl ester; ECM, extracellular matrix.

substrates in vitro. JG22 and CSAT apparently recognize the same antigen, a 140-kD complex of proteins localized in the region of cell-substratum attachment (6, 11, 16). The 140-kD complex has recently been shown to co-distribute with both α -actinin and fibronectin, which suggests that the antigens may represent a cell surface linkage between ECM molecules and the cytoskeleton (6).

In this study I examined some effects of the monoclonal antibody JG22 on neural crest cells in vitro and in vivo. To test the effects of JG22 in vivo, a novel perturbation approach has been developed in which antibody-containing medium together with the hybridoma cells themselves are microinjected onto neural crest pathways. The hybridoma cells are labeled by a new fluorescent staining method that renders the cells identifiable in living preparations and histologically processed tissue sections. Both in tissue culture and in the embryo, JG22 affects the adhesion and migration of neural crest cells. In contrast, two other monoclonal antibodies, 1-B and 1-N, that bind to bands of the 140-kD complex precipitated by JG22, affect neither cell adhesion or neural crest migration. The results suggest that the adhesive interaction blocked by JG22 is important for normal neural crest migration.

MATERIALS AND METHODS

Neural Crest Cultures

Primary cultures of neural crest cells were prepared from the neural tubes of Japanese quail embryos (*coturnix coturnix japonica*) (7, 8). Embryos were incubated for 48 h at 38°C, at which time their developmental age was comparable to chick stages 13–15 (12). The region of the trunk consisting of the six to nine most recently formed somites and some unsegmented mesenchyme was dissected away from the embryo. The neural tubes were isolated from surrounding tissues by proteolytic digestion with 150 U/ml of collagenase (CLS II; Cooper Biomedical Inc., Malvern, PA) for 15 min at 4°C and for an additional 6–9 minutes at 37°C. The reaction was stopped with complete tissue culture medium of Eagle's minimum essential medium containing 15% horse serum and 10% chick embryo extract, since the serum components contain endogenous protease inhibitors. The isolated neural tubes, with notochords sometimes attached to the ventral surface, were rinsed in complete medium and then plated onto fibronectin- or laminin-coated substrates. After 2–3 h, neural crest cells begin migrating away from the dorsal aspect of the neural tube and form a monolayer of cells on the culture dish. At this time, the cultures were fed complete medium (Eagle's minimum essential medium, 10% embryo extract, 15% horse serum). In some cases, Eagle's minimum essential medium, a defined medium lacking serum and embryo extract, was substituted for complete medium during rinsing, plating, and feeding of the cells to eliminate any possible effects of serum or embryo extract components. Most cultures were used for adhesion assays 24 h after plating. In those cultures maintained for longer periods, neural tubes were scraped away after 24 h, leaving a monolayer of neural crest cells. Cultures were fed fresh medium every other day. For some experiments, somites isolated in the same manner as the neural tubes described above were plated onto fibronectin- or laminin-coated substrates.

Attachment of Extracellular Matrix Molecules to Tissue Culture Plates

To prepare fibronectin substrates, 35-mm petri plates were incubated with 25 μ g/ml of fibronectin (Melyo Laboratories, Inc., Springfield, VA) in phosphate-buffered saline (PBS) for 10 min. After removal of the fibronectin solution, the plates were air-dried. Laminin-coated dishes were prepared by incubating the petri dishes with 0.5 mg/ml poly-D-lysine for 30 min at 37°C; the plates were washed four times with PBS and then incubated overnight with 20 μ g/ml of laminin (kindly provided by Dr. L. Reichardt, University of California, San Francisco, CA). Before plating of neural tubes, the laminin solution was removed and the plates were washed with PBS.

Hybridoma Cells

JG22 hybridoma cells were kindly provided by Dr. David Gottlieb (Washington University, St. Louis, MO). 1-B and 1-N hybridoma cells were a generous

gift from Dr. Clayton Buck (Wistar Institute, Philadelphia, PA). The cells were maintained in 75- or 250-ml tissue culture flasks in Dulbecco's modified eagle's medium containing 15% fetal bovine serum.

Labeling of Hybridoma Cells

Both 5- and 6-carboxyfluorescein diacetate succinimyl ester and fluorescein-5-isothiocyanate diacetate (Molecular Probes Inc., Junction City, OR) were used to vitally dye cells. These dyes remain visible within the cells after fixation, paraffin embedding, and serial sectioning. Most experiments were performed with carboxyfluorescein diacetate succinimyl ester (CFSE) because this proved to be the more intense and long-lived marker. A 10 mM stock solution of CFSE was prepared in dimethyl sulfoxide and stored at 4°C. For cell labeling, the CFSE working solution was prepared by diluting the stock solution 1:300 in PBS, pH 7.4. Hybridoma cells were centrifuged and the medium was removed. The cells were then suspended in CFSE working solution and incubated at 37°C for 30 min. The labeled hybridoma cells were centrifuged, resuspended in medium containing antibodies, and microinjected into embryos.

Concentration of Antibodies

The culture medium was removed from the hybridoma cells and placed in Amicon microcentrifuge filters with a 30,000-mol-wt cut-off (Amicon Corp., Scientific Systems Div., Danvers, MA). The medium containing the mouse IgG's was centrifuged at 4,800 g for 2–3 h. This procedure yielded a 20–30 fold concentration of antibody in the retentate.

Preparation of the Host

White leghorn chicken embryos were incubated at 38°C. Embryos at developmental stages 8–10 were used for injections into the mesencephalic region, and those at stages 12–15 for injections in the trunk. The eggs were washed with 70% ethanol, windowed, and stained with an injection of India ink (Pelikan, Hanover, FRG) under the blastodisk (23). The vitelline membrane was removed over the injection site. After injection, the embryo was closed with adhesive tape and returned to the incubator until the time of fixation.

Microinjection of Antibody and Hybridoma Cells into Chick Embryos

Microinjections were performed using a previously described injection technique (3, 4). The hybridoma cells and the antibody containing medium were backfilled into fine glass micropipettes. The tip of the pipette was broken off to produce an opening of 20–30- μ m diam. The injection micropipette was mounted on the electrode carrier of a micromanipulator and connected to an air pressure source. The manipulator was then used to insert the micropipette into the appropriate embryonic site, either lateral to the neural tube in the mesencephalon or into the embryonic somites in the trunk. The hybridoma cells and antibodies were expelled with a pulse of pressure and the injection needle was withdrawn. Approximately 4 nl was injected into the mesencephalic region, and ~0.5 nl into the trunk somites.

Histological Procedures

PARAFFIN SECTIONS: Embryos were fixed for 2 h in Zenker's fixative and placed first into running water for 2 h, and then into 70% ethanol. The embryos were dehydrated, embedded in paraplast, and serially sectioned at 10 μ m. Sections were mounted on albuminized slides and dried for several hours before antibody staining.

CRYOSTAT SECTIONS: Embryos were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1.5 h, rinsed in PBS, and dehydrated through a graded series of ethanols. Embryos were then placed in absolute ethanol for 48 h. In some cases, the paraformaldehyde fixation were omitted and embryos were placed directly into ethanol. The embryos were hydrated in PBS and placed first into 5% sucrose containing 0.01% azide for 2–4 h and then into 15% sucrose at 4°C overnight. They were placed in 7.5% gelatin/15% sucrose solution at 37°C for 2–3 hours, oriented, and frozen in Tissue Tek OCT (Miles Laboratories Inc., Elkhart, IN) in liquid nitrogen. Frozen sections were cut at 10 μ m on an A/O Histostat Cryostat (Reichardt Scientific Instruments, Buffalo, NY).

Immunofluorescent Staining

Slides were scanned for fluorescently labeled hybridoma cells which are readily visible under epifluorescent illumination within paraffin sections. The sections containing hybridoma cells and neighboring sections were then stained

with a monoclonal antibody HNK-1 that recognizes neural crest cells. HNK-1 is a mouse IgM that stains migrating avian neural crest cells, as well as neurons, glia, and a subpopulation of leukocytes (26). The cell surface antigen recognized by HNK-1 is the same antigen recognized by NC-1 (26). The sections were deparaffinized in histosol, taken through a graded series of alcohols, and placed into PBS. They were then incubated with HNK-1 (diluted 1:30) in a humidified chamber at room temperature overnight. The slides were washed in PBS and incubated with a rabbit antibody against mouse IgM's for 1 h at room temperature. The sections were then washed and incubated with a fluorescein-conjugated goat antibody against rabbit IgG's. After sections were rinsed in PBS, a few drops of fresh PBS were added; the slides were covered with coverslips and observed under a Zeiss epifluorescence microscope.

Because JG22 is an IgG whereas HNK-1 is an IgM, it is possible to perform double labeling experiments using differently labeled second antibodies. Cryostat sections of chick embryos were prepared. Sections were incubated overnight in a humidified chamber with medium containing JG22 and with HNK-1 (1:30). The sections were washed in PBS, incubated with a rabbit anti-mouse IgM for 1 h, washed, and stained with rhodamine-conjugated goat antibodies against mouse IgG (1:25) and fluorescein-conjugated goat antibodies against rabbit IgG's. After they were rinsed and covered with coverslips, sections were observed under epifluorescent illumination. Data were recorded photographically and on videotapes using a Silicon Intensified Target (SIT; RCA, Lancaster, PA) image intensifying video camera and a video recorder.

Quantitative Analysis of Neural Crest Inhibition

To quantitate the volume occupied by neural crest cells, serial sections through the injected embryos were examined under the fluorescence microscope. Those areas containing neural crest cells could be recognized by their HNK-1 fluorescence. The main site of injection was easily identifiable since it contained the highest concentration of hybridoma cells. Videotape recordings were made of sections throughout the mesencephalon anterior and posterior to the injection site, or through one or more somites anterior and posterior to the injection site in the trunk. To quantitate the effects of JG22 on neural crest migration, tracings that outlined the area occupied by neural crest cells were prepared off of the video screen for every section along $\sim 220 \mu\text{m}$ of the anterior-posterior extent of the embryo centered on the injection site. The surface area of each tracing was determined using an Orthoplex Coordinate Sensor Type 3825-1 (Elographics Inc., Oakridge, TN) and a Wang series 600 computer (Wang Laboratories, Inc., Lowell, MA). For each side of the embryo, the surface areas from all tracings were summed. Because the volume equals the surface area times the section thickness, the ratio of the summed areas reflects the fraction of the volume occupied by neural crest cells on the injected versus the control side.

RESULTS

JG22 is an antibody made against chick skeletal muscle cells (11) that recognizes a cell surface molecule involved in muscle cell attachment to tissue culture substrates. In the presence of the antibody, muscle cells undergo rapid changes in morphology and sometimes detach from the substrate, whereas fibroblasts in the same cultures remain unaffected. The effects of JG22 on neural crest adhesion in vitro and neural crest migration in vivo are described below.

Effects of JG22 Antibody on Neural Crest Cells In Vitro

Neural crest cell cultures were prepared from quail neural primordia as described (see Materials and Methods) and plated onto fibronectin or laminin-coated petri dishes. The culture medium was removed and replaced with medium conditioned by JG22 hybridoma cells. Control cultures were fed either medium alone (Dulbecco's modified Eagle's medium plus 15% fetal bovine serum) or medium containing the 1-B or 1-N control antibodies. The control antibodies bind to bands 1 and 3, respectively, in the 140-kD complex recognized by JG22 and CSAT (Buck, C., personal communication). Neural crest cells are typically grown in a rich culture medium containing horse serum and embryo extract.

For some experiments, neural crest cells were plated and grown in medium without serum or embryo extract to eliminate any possible contributions of horse serum or embryo extract components to the tissue culture substrate. Identical results were observed in both types of media.

JG22 antibodies caused rapid and profound changes in the morphology of neural crest cells grown on fibronectin or laminin substrates. In neural crest cultures 24 h after plating, most neural crest cells rounded up and became phase bright within minutes after addition of the antibody (Fig. 1). Some of the rounded cells detached from the dish, whereas others remained tenuously attached. In addition, many neural crest cells tended to form small aggregates. Similar effects were observed in cultures of various ages ranging from 6 h to 8 d after plating. In contrast, other cell types such as neural tube cells, notochord cells, or somite cells included on the same culture dish underwent little or no morphological change. In some cases, the edges of the epithelial sheets formed by the cultured neural tube cells did appear to roll up slightly. Cells in control cultures fed fresh culture medium, medium containing 1-B, or medium containing 1-N antibodies were unperturbed.

JG22 Antigen Is Present on Migrating Neural Crest Cells

Cryostat sections of chicken embryos were double labeled with JG22 antibody (a mouse IgG) and with HNK-1 antibody (a mouse IgM). HNK-1 was used to identify neural crest cells (26). JG22 antigen was detected on the surface of migrating neural crest cells, in both the trunk and the mesencephalic region (Fig. 2). In addition, JG22 staining was present around the basal surface of the neural tube, notochord, and myotome. A detailed description of the distribution pattern of the JG22 antigen in early embryos will be described elsewhere (Krotoski, D., E. Montgomery, and M. Bronner-Fraser, manuscript in preparation). It is interesting that JG22 antibody did not cause detachment of neural tube, notochord, or somite cells from fibronectin or laminin substrates in vitro. These results suggest that these tissues may have some adhesive mechanisms in addition to the antigen recognized by JG22.

Labeling of JG22 Hybridoma Cells

The purpose of injecting hybridoma cells into the developing embryos was twofold: to serve as a convenient marker for the site of injection and to provide a continuous source of antibody within the embryo. To allow the cells to be located after injection, the hybridomas must be labeled with a marker that can survive the fixation and embedding procedure used to analyze the data. In addition, the dye must be nondeleterious to the function of the hybridoma cells. Of the dyes tested, CFSE was found to stain the hybridoma cells intensely both before and after fixation in 10% buffered formalin or in Zenker's fixative. The CFSE diffuses freely across the cell membrane, where intracellular esterases release the chromaphore. The negatively charged chromaphore is then retained within the cell. The CFSE remains within the labeled cells over long periods; one set of hybridoma cells was maintained in culture for 3 wk and still contained many fluorescent cells.

To assess the effects of CFSE labeling on antibody production, hybridoma cells were labeled as described in Materials and Methods, placed in fresh culture medium, and allowed

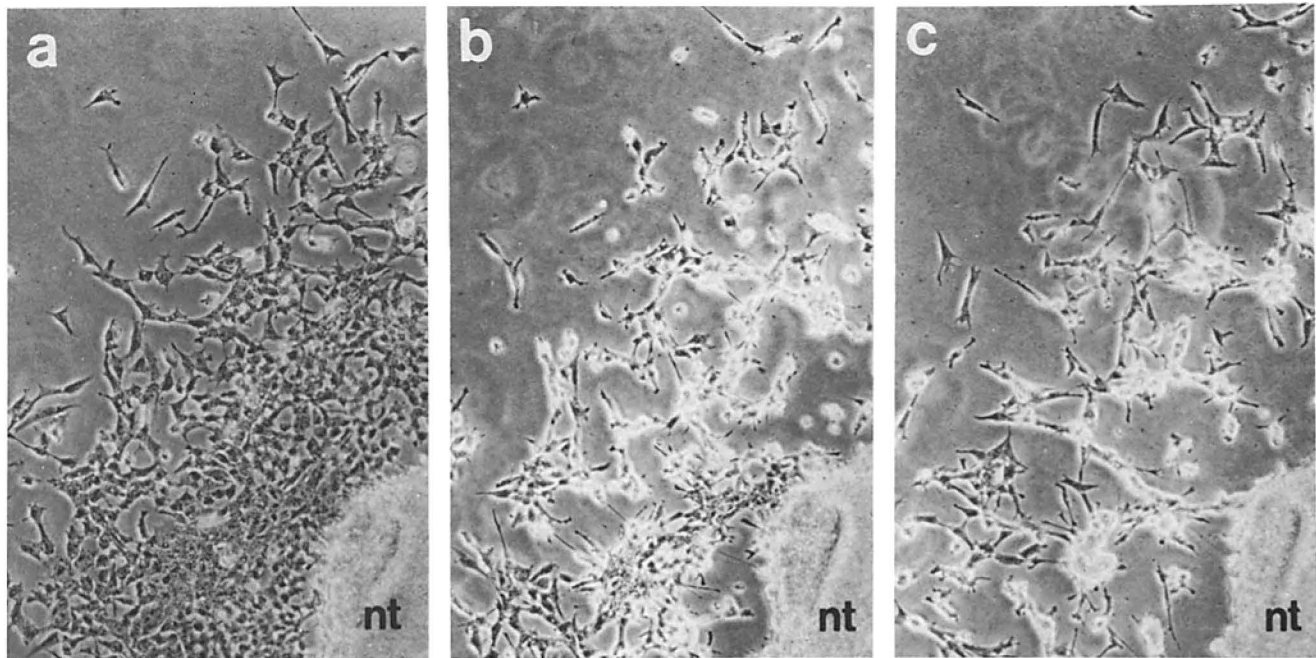


FIGURE 1 Phase-contrast photomicrographs illustrating the effects of JG22 antibody on neural crest cells in vitro. (a) A culture of neural crest cells grown on fibronectin 24 h after explantation of the neural tube (nt) onto the culture dish. The neural crest cells have emigrated from the neural tube and have formed a monolayer on the substrate. (b) The same field 15 min after addition of JG22 antibodies. Many cells have become rounded and phase bright, and often detach from the substrate; other cells form small aggregates. (c) The same field after 60 min. More cells have detached or formed aggregates. Note that the neural tube does not appear to be affected. $\times 200$.

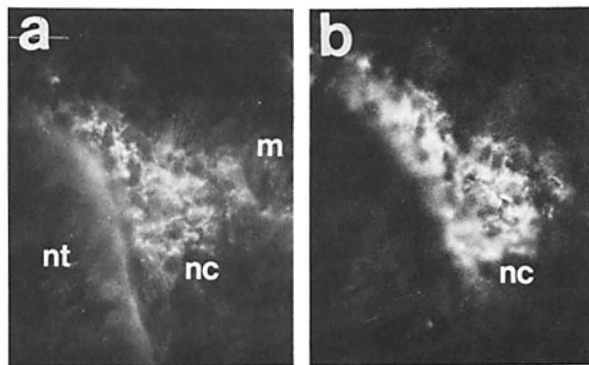


FIGURE 2 Fluorescence photomicrographs of a cryostat section through the trunk of a 2.5-d chick embryo. (a) Rhodamine immunofluorescence of JG22 staining. $\times 880$. The JG22 antigen is seen on the basolateral side of the neural tube (nt), the myotome (m), and on the surface of neural crest cells (nc). In addition, JG22 is detected around the notochord (not shown). (b) The same section visualized through a fluorescein filter set shows HNK-1 staining of neural crest cells $\times 880$.

to proliferate for 24 h. The culture medium was then removed and tested for the presence of JG22 antibodies by an assay of its effects on neural crest adhesion in vitro. The culture supernatant from the labeled cells caused rapid rounding and detachment of neural crest cells; these effects were identical in morphology and time course to those caused by JG22 medium removed from unlabeled hybridoma cells.

Effects of JG22 Antibody on Cranial Neural Crest Migration

After they were labeled, hybridoma cells were resuspended

in either fresh medium or medium that contained JG22 antibody concentrated 20- to 30-fold. The antibody and cell suspension was backfilled into a micropipette and injected into the mesencephalon during the early stages of cranial neural crest migration. Each embryo was injected lateral to the mesencephalic neural tube with ~ 4 nl cell suspension. The embryos were allowed to develop from 4 to 22 h after injection, fixed, sectioned, and stained with HNK-1 antibody to monitor the effects of the injection on neural crest migration. The labeled hybridoma cells were easily detected in fixed and antibody-stained sections because the cells have round, brightly fluorescent cell bodies, which can be visualized with several different fluorescent filter sets.

In an initial series of experiments, the effects of the JG22 hybridoma cells alone were examined; 10 embryos were injected with JG22 hybridoma cells plus fresh culture medium, without JG22 antibody. In one of these embryos, a reduction of neural crest migration was observed on the injected side relative to the control side. In the remaining embryos, few or no differences in cell numbers were noted between injected and control sides; however, a cell free zone was observed between the hybridoma cells and the neural crest cells. In the case of JG22 hybridoma cells, $78 \pm 6\%$ ($n = 6$ embryos) of the hybridoma cells were located at least one cell diameter away from the closest neural crest cell. This is significantly higher ($P < 0.025$) than in control embryos, where only $31 \pm 7\%$ ($n = 3$ embryos injected with 1-B hybridoma cells) and $41 \pm 25\%$ ($n = 5$ embryos injected with 1-N hybridoma cells) were one or more cell diameters distant from the nearest neural crest cell. These results suggest that the JG22 hybridoma cells usually affect neural crest cells only at a local level, within a few cell diameters.

In a second series of experiments, hybridoma cells were

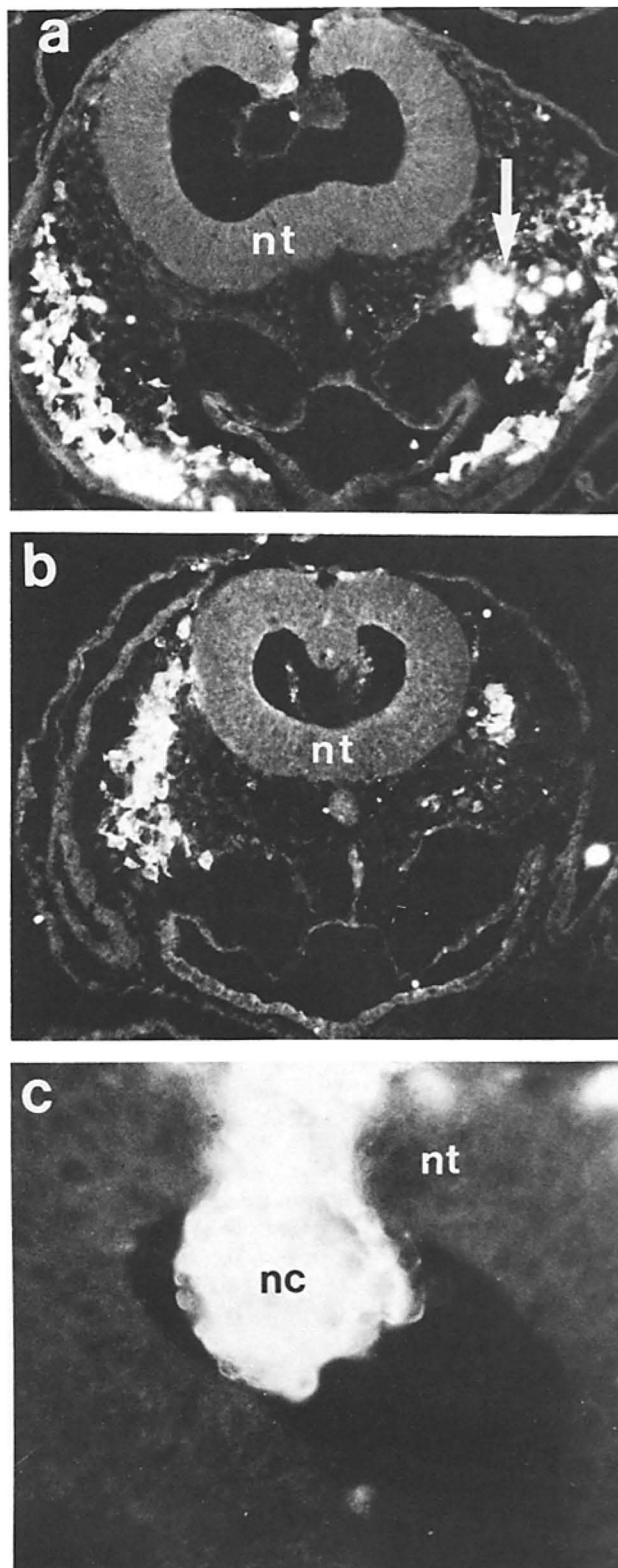


FIGURE 3 Fluorescence photomicrographs of embryos fixed 11 h after injection of JG22 antibodies and hybridoma cells into the mesencephalon. The sections were stained with HNK-1 antibodies to recognize neural crest cells. Neural crest cells in the mesencephalon normally emigrate away from the neural tube (nt) and into the mesenchyme just beneath the ectoderm. (a) A section through the main injection site, containing the highest concentration of hybridoma cells. $\times 330$. The brightly fluorescent hybridoma cells that were labeled with CFSE before injection are easily seen on the

injected together with antibody-containing medium. In addition to the cell-free space observed between most injected JG22 hybridoma cells and the endogenous neural crest cells, profound alterations in neural crest migration were observed in 34 of the 41 embryos injected with JG22 antibodies (Fig. 3). The amount of neural crest migration on the injected side appeared reduced. The neural crest cell volume was determined by measuring the area occupied by neural crest cells in each section through the mesencephalon in six randomly selected embryos. The volume on the injected side was reduced by $54 \pm 5\%$ in these animals relative to the noninjected side. Note that the calculated 54% reduction is summed over the entire mesencephalon and that individual sections may have a much larger difference in neural crest cell number between the injected and control side (Fig. 3b). The decrease in volume on the injected side probably reflects an actual decrease in numbers of neural crest cells; on both injected and control sides, neural crest cells migrate in close association to one another and the size of individual cells appears unchanged on the injected side.

In addition to reduced cell numbers, 9 of the 34 affected embryos had a lobule containing HNK-1 positive cells protruding into the lumen of the neural tube (Fig. 3c). Presumably, these stained cells are neural crest cells; HNK-1 normally recognizes neural crest cells only after they have departed from the neural tube. This build-up of neural crest cells may reflect some inhibition of normal crest emigration from the dorsal neural tube. It is interesting that the most profound alterations in neural crest migration were often observed posterior to the main injection site (i.e., the region with the highest concentration of hybridoma cells). In the anterior regions, many neural crest cells may have already left the neural tube at the time of injection. Perhaps the more caudal regions of the mesencephalon, where neural crest migration is just beginning, have the greatest sensitivity to the injected antibody.

In a few of the experimental embryos, those neural crest cells present on the injected side migrated along an aberrant pathway; instead of moving laterally under the ectoderm the cells migrated along the ventral portion of the neural tube and through the mesenchyme. In 7 of the 41 embryos, no alterations in neural crest migration were apparent. In these cases, it is possible that the antibody was diluted through leakage out of the puncture hole made by the injection needle.

Effects of 1-B and 1-N Antibodies on Cranial Neural Crest Migration

A possible complication of the antibody injection approach

right-hand side (indicated by the arrow). In the presence of JG22 antibodies, the number of neural crest cells on the injected side was markedly reduced. Quantitative reconstruction of serial sections throughout the mesencephalon of this particular embryo demonstrated a 51% reduction in neural crest cell volume on the injected side relative to the control side. (b) Another section through the posterior mesencephalon of the same embryo illustrated in a. $\times 330$. The reduction in neural crest cell number is more severe and the cells have not migrated as far ventrally on the injected side. In addition, a lobule has formed within the neural tube (nt) which contains neural crest cells (identified by HNK-1 staining). (c) Another embryo at higher magnification with a profound build up of neural crest cells (nc) in the lumen of the mesencephalic neural tube. $\times 1,200$.

for perturbing neural crest migration is that the effect produced by an antibody may be unrelated to a functional block of the antigen it recognizes. To control for such a possibility, the experiments described above were repeated using two different hybridomas clones, 1-B or 1-N, instead of JG22. 1-B and 1-N cells produce antibodies that recognize bands 1 and 3, respectively, of the complex precipitated by JG22 and CSAT (Buck, C., personal communication), but unlike JG22 and CSAT do not alter cell adhesion. In tissue culture, neither 1-B or 1-N antibodies caused detachment of neural crest cells. After the injection of 1-B ($n = 14$ embryos) or 1-N ($n = 8$ embryos) hybridoma cells and concentrated antibody solution into the mesencephalic region, no inhibition of neural crest migration and no build-up of neural crest cells within the dorsal neural tube was observed (Fig. 4). The volume occupied by neural crest cells on both the injected and control sides was quantitated in serial sections through six representative embryos. The volume on the side injected with hybridoma cells was $102 \pm 8\%$ of that on the control side for 1-B, and $98 \pm 7\%$ for the 1-N antibody. The reduction for embryos injected with JG22 hybridoma cells was 54%. Thus, introduction of IgG's and hybridoma cells that do not block the function of the 140-kD complex had no noticeable effect on the distribution of neural crest cells. These results suggest that the alterations in neural crest migration caused by JG22 result from a functional block of the 140-kD complex.

Effects of JG22 Antibody on Trunk Neural Crest Migration

To examine the affects of JG22 antibody on neural crest migration in the trunk, JG22 antibodies and labeled hybridoma cells were injected into the trunk somites. Injections were made ~10 somites anterior to the most recently formed somite. This is a level where endogenous neural crest cells are just beginning to penetrate the anterior part of the somite after the somite dissociates into the dermatome, myotome, and sclerotome (Rickmann, R., J. Fawcett, and R. Keynes, manuscript in preparation; Bronner-Fraser, M., manuscript submitted for publication). Approximately 0.5 nl was injected into each somite. Embryos were fixed and processed from 7 to 21 h after the initial injection.

As observed in the cranial region, a separation between injected JG22 hybridoma cells and neural crest cells was observed in the trunk. Other effects of JG22 on trunk neural crest migration were variable. Seven of the 28 embryos examined did have a decreased volume of neural crest cells on the injected side, though to a lesser extent than noted in the mesencephalon. A 15% reduction in neural crest cell volume on the injected side relative to the control side was determined by quantitative reconstruction of one representative embryo. In another two embryos, neural crest cells were observed migrating along aberrant pathways. In these cases, crest cells were seen invading the region around the notochord, or were seen within the myotome. In normal embryos, neural crest cells avoid the space around the notochord and do not penetrate the myotome (19). In 19 of the 28 embryos examined, no marked differences in neural crest migration were observed on the injected side relative to the control side.

DISCUSSION

This study examines the effects of the JG22 antibody on neural crest cell adhesion in tissue culture and in the embryo.

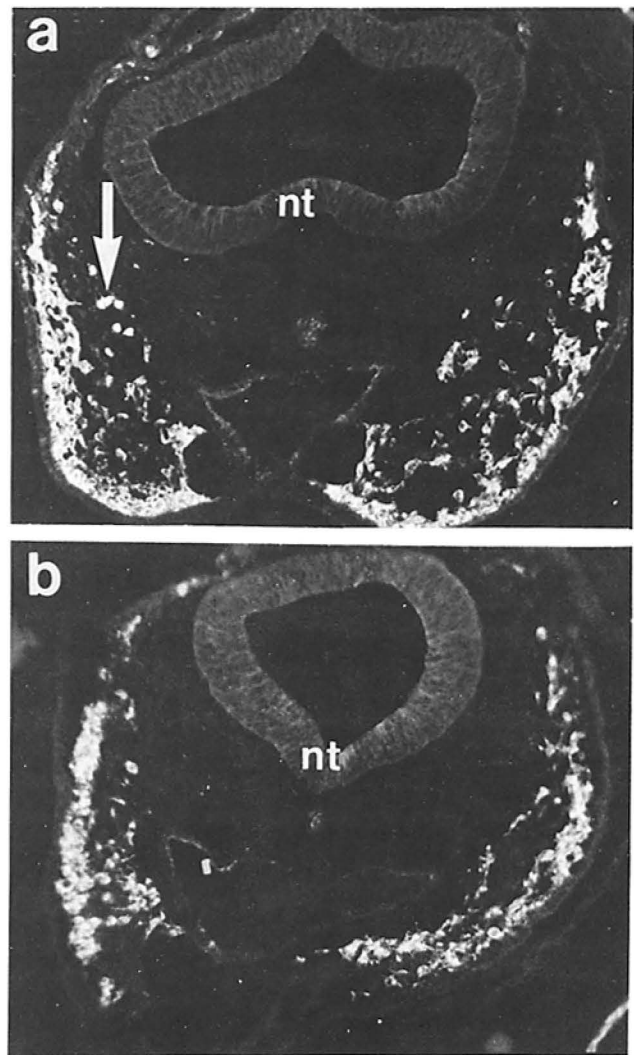


FIGURE 4 Fluorescence photomicrographs of a control embryo fixed 11 h after injection of 1-N hybridoma cells and antibodies into the mesencephalon. The sections were stained with HNK-1 antibodies to recognize neural crest cells. (a) A section through the main injection site containing the highest concentration of hybridoma cells, indicated by the arrow. $\times 260$. (b) Another section through the posterior mesencephalon of the same embryo illustrated in a. $\times 260$. After injection of control hybridoma cells and antibodies, no differences in the number of neural crest cells were noted between the injected side and the control side. Quantitative reconstruction of serial sections throughout the mesencephalon of this particular embryo demonstrated that the neural crest cell volume on the injected side was 96% of that on the control side. In contrast, embryos injected with JG22 antibodies and hybridoma cells had a 50% reduction in neural crest cell volume (compare Fig. 3). nt, neural tube.

The antibody, originally found to perturb adhesion of skeletal muscle cells in vitro (11), was discovered to cause rounding and detachment of neural crest cells plated on both fibronectin and laminin tissue culture substrates. Control antibodies (1-B and 1-N) that bind to bands of the 140-kD complex precipitated by JG22 (Buck, C., personal communication) had no affect on neural crest cell adhesion. In frozen sections of young chick embryos, the JG22 antigen is present on the surface of neural crest cells and is also found on the basal surface of the neural tube, myotome, and notochord. The latter tissues, however, were not affected in vitro by the

addition of JG22 antibodies.

To examine the effects of this antibody on neural crest cells *in vivo*, a new approach for perturbing embryonic cell migration was developed. JG22 antibodies together with the hybridoma cells were microinjected onto neural crest pathways. The hybridoma cells were stained with a long-lasting fluorescent marker that is nondeleterious and has the advantage of being detectable in fixed histological sections. The labeled hybridoma cells serve both as markers for the exact locus of the injection site and as a continuous source of antibody. When JG22 antibodies and hybridoma cells were microinjected into the mesencephalic neural crest pathway, the following changes were observed: (a) neural crest migration was reduced in 83% of the injected embryos; (b) a build-up of neural crest cells within the lumen of the neural tube occurred in 26% of the affected embryos; (c) neural crest cells migrated along aberrant pathways in 6% of the affected embryos; and (d) a space of one or more cell diameters was seen between the neural crest cells and most of the JG22 hybridoma cells in all injected embryos. Control injections performed with the 1-B or 1-N antibodies and hybridoma cells did not cause alterations in neural crest migration. These findings suggest that the observed effects are caused by functional block of the 140-kD complex recognized by JG22. In the trunk region, the effects of JG22 were far less reproducible and consistent for the antibody titer and injected volumes used in this study. In a few cases, the numbers of neural crest cells were somewhat reduced, and in other cases, the crest cells moved in aberrant directions to invade the perinotochordal space and the myotome. Normally, no crest cells are seen within these spaces (Bronner-Fraser, M., manuscript submitted for publication; 19).

A quantitation of neural crest cell volume of the injected side relative to the noninjected side of the embryos demonstrated a 50% reduction of cranial neural crest cell migration after injection of JG22 antibodies; no reduction was observed in control embryos injected with 1-B or 1-N antibodies and hybridoma cells. The serial reconstructions used in this study demonstrate that simple quantitation can provide useful information in systems as complicated as neural crest migration. Such methods provide a global picture of the effects produced by perturbation techniques. In contrast, examination of individual sections may prove misleading since no section can be truly representative and the effects may be masked or amplified by the exact plane of section.

Several possible explanations may account for the apparent reduction in the number of neural crest cells after injection of JG22. For example, the antibody may diminish the ability of neural crest cells to emigrate from the neural tube. In the absence of compensatory hyperplasia, this would result in a decrease in the total number of neural crest cells on the injected side. In support of this idea, JG22 often led to a build-up of neural crest cells within the lumen of the neural tube. Alternatively, JG22 may affect the neural crest cells only after their departure from the neural tube, by altering either their ability to proliferate or their survival. Future experiments that examine the effects of JG22 on neural crest cells *in vitro* may distinguish among these alternatives.

The results indicate that the cell surface antigen recognized by JG22 is important for the normal migration of neural crest cells in the cranial region since the amount of neural crest migration is reduced in the presence of the antibody. Neural crest migratory routes in the mesencephalon are rich in fibro-

nectin (10). Therefore, it is likely that JG22 perturbs the normal adhesion of neural crest cells to a fibronectin-rich matrix and that this adhesion is important for normal cell migration. It is interesting that a similar inhibition of neural crest migration was observed by Boucaut et al. (2) after a synthetic decapeptide that appears to contain the cell recognition sequence of fibronectin (21, 27) was injected into the mesencephalic neural crest pathway. These investigators observed a bilateral reduction in numbers of cranial neural crest cells and a build-up of neural crest cells within the lumen of the neural tube (2). Presumably, both the decapeptide and the JG22 antibody alter the adhesion of neural crest cells to fibronectin. Support for this idea comes from a recent study on chick embryo fibroblasts, which demonstrates that the JG22 antigen co-distributes with extracellular fibronectin and intracellular α -actinin (6). It is surprising that JG22 also affects adhesion of neural crest cells to laminin-coated substrates, which suggests that the 140-kD complex may not be completely specific for fibronectin. Studies that show that the JG22 antigen is present on the basolateral membrane surface of the intestinal epithelium (5) suggest that the 140-kD complex may be associated with laminin *in situ*. In contrast to JG22, the synthetic peptides containing the fibronectin cell recognition site do not appear to affect cell adhesion to laminin substrates.

Fibronectin is present in both the trunk and cranial regions of the embryo during neural crest migration (10, 24). At the concentrations used in this study, the effects of JG22 antibody in the trunk were less dramatic than those observed in the cranial region, even though the antibody and hybridoma cells were injected into regions of active neural crest migration. This may result from the fact that a much smaller volume of antibody can be conveniently introduced into the trunk compared to the cranial regions. The smaller volume occupied by neural crest cells in the trunk might be expected to partially compensate for the smaller injection volume; however, the results show that the trunk injections are less effective. Perhaps the smaller injected volume leads to a greater proportional leakage of antibody out of the injection site, thereby lowering the effective antibody concentrations. In addition, the tissues surrounding the injection site may absorb more JG22 antibody in the trunk than in the cranial region leaving less antibody free to interact with the neural crest cells. Another possible and more intriguing explanation is that the extracellular matrix may be different or more complex in the trunk than in cranial regions. Though neural crest cells in the mesencephalon seem to follow fibronectin-rich pathways, recent studies indicate that most neural crest cells in the trunk migrate through the anterior portion of each somite, which contains little or no fibronectin (Rickmann, R., J. Fawcett, and R. Keynes, manuscript in preparation). If neural crest cells migrate along ECMs, additional species of matrix molecules must be present in the trunk. Thus, the adhesive interactions perturbed by JG22 may not be the dominant cell surface-ECM interaction involved in trunk neural crest migration. We are currently examining these different possibilities.

It is interesting to note that a number of embryonic cell types including the neural tube, notochord, and myotome have the JG22 antigen on their surface; however, the adhesion of these cells to tissue culture substrates is not perturbed in the presence of antibody. Decker et al. (9) have reported similar findings using the CSAT antibody. They detected the

140-kD complex on the surface of skeletal and cardiac muscle as well as on tendon, skeletal, dermal, and cardiac fibroblasts. In contrast, the antibody caused detectable morphological changes only on skeletal muscle and cardiac fibroblasts. They suggest that there may be an adhesive hierarchy in different cell types with the CSAT/JG22 antigen appearing as the lowest common denominator. This idea receives support from the finding that JG22 antibody prevents the initial adhesion of chick embryo fibroblasts to fibronectin substrates (6) but does not affect cell morphology when added after cell attachment and spreading have taken place.

The present study describes a new antibody perturbation technique in which both the antibody and hybridoma cells are introduced into embryos. This perturbation approach may be generally applicable in studies elucidating the roles of certain antigens in early embryogenesis. In this article a novel cell labeling technique is also described which allows long-term identification of implanted cells. This labeling method may provide a generally useful marker for intra- and interspecific grafts (Bronner-Fraser, M., and S. Fraser, manuscript in preparation), as well as for in vitro experiments in which differentially labeled tissues are brought into contact. By use of this approach, the antibody JG22 was found to cause profound alterations in cranial neural crest migration. This probably results from the binding and functional inactivation by JG22 of a cell surface complex involved in recognition of fibronectin (6). The results suggest that this cell surface molecule may be important for normal cranial neural crest migration. The results on neural crest migration are too incomplete to support the naive suggestion that interactions between a single cell surface component and an ECM molecule are uniquely responsible for the guidance of a complex developmental process such as neural crest migration. Rather, the data remain consistent with the presence of a number of molecules; these may function synergistically such that perturbation of one or more of their interactions would alter normal morphogenesis.

I thank Drs. Scott Fraser and James Coulombe for their helpful comments on the manuscript, and Georgia Guillory and Elizabeth Montgomery for their excellent technical assistance.

This work was supported by U. S. Public Health Service grant HD-15527-01 and by a Basic Research Grant from the March of Dimes Birth Defects Foundation.

Received for publication 22 March 1985, and in revised form 27 April 1985.

Note Added in Proof: Since this manuscript went to press, analogous experiments were performed using CSAT (16; kindly provided by Dr. C. Buck), which binds to the same 140-kD complex recognized by JG22. The preliminary results suggest that CSAT causes alterations

in neural crest adhesion in vitro and migration in vivo similar to those observed after exposure to JG22.

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